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Synthesis of silver nanoparticles using 3,5-di-*t*-butyl-4-hydroxyanisole from *Cynodon dactylon* against *Aedes aegypti* and *Culex quinquefasciatus*

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ABSTRACT

Emergence of resistance among mosquitoes is recent problem. In the present study, was mosquito larvicidal activity of synthesized silver nanoparticles (Ag NPs) 3,5-di-*t*-butyl-4-hydroxyanisole isolated from *Cynodon dactylon* against the first to fourth instar larvae dengue vector, *Aedes aegypti* and filariasis vector, *Culex quinquefasciatus* (Diptera: Culicidae) was carried out. The synthesized Ag NPs were characterized by UV, XRD and HR-TEM. The HR-TEM analyses were spherical shaped. The highest mortality of synthesized Ag NPs tested against first to fourth instars of *A. aegypti* (LC_{50} = 2.50, 2.78, 3.02, 3.05 μg/mL; LC_{90} = 8.28, 7.47, 8.13, 8.74 μg/mL) and *C. quinquefasciatus* (LC_{50} = 2.75, 3.00, 3.21, 3.48 μg/mL; LC_{90} = 6.45, 7.72, 11.06, 12.83 μg/mL), at 10 μg/mL, respectively. Synthesized Ag nanoparticles were effective as potential anti larvicidal agent against *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes.

Introduction

Mosquitoes are well known group of insects, which transmit many dreadful diseases causing serious health problems to human beings. Controlling of these vectors has been achieved for a long time, using synthetic chemicals. Dengue, ziga viruses is found in tropical and sub-tropical climates worldwide; its major vector is the mosquito *Aedes aegypti* (WHO, 2015). Recently, dengue transmission has strongly enlarged in urban and semiurban areas, becoming a major global public health concern and over 3.9 billion people, in 128 countries, are at risk of infection with dengue (Bhatt et al., 2013). Microfilariae are transmitted to humans by different mosquitoes like *Culex* species, with a special reference to *Culex quinquefasciatus*. Lymphatic filariasis (elephantiasis) infects about 120 million people in tropical areas of Africa, India, South-East Asia (WHO, 2015).

Nanoparticle synthesis is currently a significant area of research, searching for an eco-friendly approach and green materials for current scenario. Velayutham and Ramanibai (2016) tested the synthesized Ag NPs using isoamyl acetate isolated from *Annona squamosa* against *A.aegypti* and *C. quinquefasciatus*. Synthesized silver nanoparticles using 2,7.bis [2-[diethylamino]-ethoxy] fluorence isolated from the *Melia azedarach* leaves against *A. aegypti* and *C. quinquefasciatus* (Ramanibai and Velayutham, 2015). Suganya et al. (2014) examined the larvicidal potential of solvent leaf extracts of *Leucas aspera* and synthesized Ag NPs using it against fourth instar larvae of *A. aegypti*. Mondal et al. (2014) investigated the bioactive components present in the root extract of *Parthenium hysterophorus* plant used for the biosynthesis of Ag NPs and analyzed the larvicidal effects of the extract as well as Ag NPs on *C. quinquefasciatus*.

Cynodon dactylon (Bermuda grass), a native grass of North Africa, Asia, Australia and southern Europe, belongs to the family Poaceae. *C. dactylon* is said to have many medicinal properties including antimicrobial and antiviral properties (Singh et al., 2008). Whole green plant aqueous extract of *C. dactylon* was used to study the diabetes induced oxidative stress of diabetic rats (Prashant Kumar et al., 2010). The constituents reported in this plant are cynodin, hydrocyanic acid, triticin, proteins (Kao et al., 2005), carbohydrates, β-carotene and minerals like calcium, phosphorous, iron and potassium (George et al., 1997). In the present study, synthesized silver nanoparticle using 3,5-di-*t*-butyl-4-hydroxyanisole isolated from *C. dactylon* leaf ethyl acetate against mosquito larvae.

Materials and methods

Plant collection and preparation of crude extracts

The fresh and healthy leaves of *C. dactylon* were collected during the year 2014 from our University campus. The plant has been authenticated by Dr. P. Jayaraman, plant taxonomist, Chennai, India. The leaves were washed with tap water and shade-dried at room temperature 27 °C for two weeks and were coarsely powdered in a powdering machine. One kilogram powder of plant leaves was extracted sequentially with hexane, chloroform, ethyl acetate and methanol at room temperature after 48 h of soaking in an aspirator bottle with the respective solvents. The extracts

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were filtered through Whatman No. 1 filter paper and condensed by vacuum rotary evaporator. The crude extracts were stored at 4 °C until used.

Isolation of active compounds

Fifteen grams of crude ethyl acetate extract *C. dactylon* leaves were subjected to silica gel (60–120 and 230–400 mesh, Acme Synthetic Chemicals, Maharashtra, India) chromatography and eluted with hexane containing increasing amounts of ethyl acetate. Fractions were monitored by TLC using precoated silica gel plates (Merck silica gel 60 F254, 0.25 mm thick) and the spots were visualized under UV (254 and 366 nm) followed by spraying with Panchal-D (Ceric ammonium sulphate (3.6 g), ammonium hepta molybdate (4.2 g), Con. H_2SO_4 (6.2 mL) in 100 mL of water) is a spraying reagent for TLC and vanillin–sulfuric acid spray reagent.

The following ratios of mobile phase were used for fractionation: hexane (100%); hexane and ethyl acetate (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50). Ten fractions were collected and concentrated; fraction five showed a single spot on TLC which was found to be a pure compound.

Molecular analysis of active fraction

Column fraction five was a single spot identified in the TLC profile which were also exhibiting higher larvicidal activity was further characterized using ^{1}H NMR (at 23 °C in $CDCl_3$ using a Burker 300 MHz spectrometer), ^{13}C NMR (Burker 300, 75 MHz spectrometer in $CDCl_3$ as solvent). The molecular structure was predicted for the fractions of antifeedant substance based on the spectrum of infrared spectroscopy and ^{1}H and ^{13}C NMR.

Synthesis of silver nanoparticles

Aqueous solution 0.5 mM of silver nitrate ($AgNO_3$) was freshly prepared with Milli Q water and used for the synthesis of Ag nanoparticles. 13 mL of hydroxyanisole was added to 87 mL of 0.5 mM $AgNO_3$ solution. The fully reduced solution was centrifuged at 10,000 rpm for 30 min. The supernatant liquid was discarded and the pellet obtained was redispersed in deionized water. The centrifugation process was repeated three times to wash off any absorbed substances on the surface of the silver nanoparticles.

Characterization of synthesized Ag NPs

The quantitative measurements of the synthesized NPs were analyzed in a Perkin-Elmer model (UV-1800) UV–vis double beam spectrophotometer from 300 to 600 nm, at the resolution of 1 nm. The bioreduction of silver ions in aqueous solution as a function of time was monitored by periodic sampling of aliquots (0.2 mL) of the suspension, then diluting the samples with 2 mL deionized water and subsequently measuring the UV–vis spectra of the resulting diluents. XRD studies, dried nanoparticles were coated on the XRD grid, and the spectra were recorded using Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with CuKα 1 radiation. The size, morphology and crystalline of both Ag NPs were also investigated using HR-TEM (High Resolution Transmission Electron Microscopy). Samples for TEM measurements were prepared by placing a drop of NP solution on the graphite grid and drying it in vacuum. Transmission electron micrographs were taken using JEOL JEM-2100F operated at an accelerated voltage of 200 kV and an ultra high-resolution pole piece.

Larvicidal activity

Mosquito *A. aegypti* larvae were collected from water tank, broken bottles, small water courses and *C. quinquefasciatus* larvae were from drainage, septic tank, cock pits, polluted water area of Tambaram, Chennai and Tamilnadu. They were maintained at the temperature of 28 ± 2 °C and 80 ± 10% RH (relative humidity) under the 12-hour light and dark photoperiod cycle. The larvae were fed dog biscuit and a brewer's yeast powder mixture 3:1 ratio is used in the laboratory. After five days, adult male mosquitoes were fed a 10% of sucrose solution. The emerging female mosquitoes obtain blood meal from white albino rat for 2–3 h for eggs production Kamaraj et al. (2008). The larvae of *A. aegypti* and *C. quinquefasciatus* were collected from the insect rearing cage and identified in entomology research institute, Loyoa College in Chennai. The larvicidal activity was assessed by the procedure of WHO (1988), minor modifications. The Fr.1 to 10, 0.5 mM solution and Synthesized Ag NPs toxicity test was performed by placing 20 mosquito larvae into 200 mL tap water in a 250 mL beaker (Borosil). Stock solution preparation was Fr.1 to 10 (10 mg) and synthesized Ag NPs (5 mg) was first dissolved in 1 L of Milli Q water. From the stock solution, the Fr.1 to 10 and 0.5 mM solution desired concentrations of (5, 10, 25, 20 and 25 ppm and 20, 40, 60, 80 and 100 μg/mL), The synthesized Ag NPs solutions were diluted using Milli Q water as a solvent according to the desired concentrations (2, 4, 6, 8 and 10 μg/mL). Each test included a set control group (distilled water) with three replicates for each individual concentration. Mortality was assessed after 24 h to determine the acute toxicities on all instars larvae of *A. aegypti* and *C. quinquefasciatus*.

Data analysis

The larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit values were calculated by using the software (Stat Plus: mac) developed by Reddy et al. (1992).

Results and discussion

In the present study, the percent mortality was observed in the Fractions 1 to 10 against first to fourth instar larvae of *A. aegypti* and *C. quinquefasciatus* at 25 ppm. The results showed that the optimal hours for measuring the percent mortality were 100, 100, 90, 70 and 45; 100, 100, 80, 70 and 42; 100, 90, 70, 60 and 40; 100, 85, 65, 46 and 35 against 1st, 2nd, 3rd and 4th instar larvae of *A. aegypti* and 100, 95, 75, 50 and 45; 100, 95, 70, 50 and 45; 100, 90, 65, 45 and 40; 100, 85, 65, 45 and 35 against 1st, 2nd, 3rd and 4th instar larvae of *C. quinquefasciatus* at 24, 18, 12, 6 and 1 h, respectively. The lethal effect of first to fourth instars larvae *A. aegypti* (LC_{50} values of 5.74, 6.14, 7.07, 18.51 ppm; LC_{90} values of 12.70, 15.50, 21.25, 23.99 ppm) (Table 1) and *C. quinquefasciatus* (8.58, 8.62, 10.28, 17.18 ppm; LC_{90} values of 19.93, 21.01, 23.77, 24.79 ppm), respectively (Table 2). However, in the present study higher mortality was observed in all the larval stages of *A. aegypti* and *C. quinquefasciatus* Fraction 5.

Ramanibai and Velayutham (2015) reported the fluorenced compound isolated from *Melia azedarach* against third instar larvae of *A. aegypti* (LC_{50} = 7.94, LC_{90} = 23.82 ppm) and *C. quinquefasciatus* (LC_{50} = 13.58, LC_{90} = 40.03 ppm). Isoamyl acetate isolated from *C. dactylon* against first to fourth instars larvae of *A. aegypti* the values of LC_{50} = 10.29, 10.72, 11.09, 11.77 ppm; LC_{90} = 31.22, 32.19, 36.14, 34.20 ppm and *C. quinquefasciatus* LC_{50} = 10.59, 11.10, 11.90, 12.71 ppm; LC_{90} = 32.11, 35.12, 37.48, 42.17 ppm (Velayutham and Ramanibai, 2016). Nikkon et al. (2010) investigated the β-amyrin isolated frome stem of *Duranta repens* against first to fourth instars larvae of *C. quinquefasciatus* with LC_{50} values of 7.75, 16.11, 28.63 and 26.53 ppm. Ethyl acetate extracts of *C. dactylon* tested caused mortality among the larvae of vector mosquito species to some extent.

The silver nitrate (0.5 mM) lethal effect of first to fourth instar larvae of *A. aegypti* (LC_{50} = 60.30, 69.36, 76.96, 80.51 μg/L; LC_{90} = 227.85, 307.09, 358.94, 338.83 μg/mL) and *C. quinquefasciatus* (LC_{50} = 68.28, 77.63, 84.06, 91.46 μg/mL; LC_{90} = 281.74, 339.38, 335.40, 381.46 μg/L) (Table 3). Jayaseelan and Rahuman (2012) has been reported the 1 mM $AgNO_3$ solution tested larvae of *Hyalomma anatolicum anatolicum*

Table 1
Mosquito larvicidal activity of *C. dactylon* fractions *Aedes aegypti.*

Extracts	Larvae instars	LC_{50}ppm (LCL-UCL)	LC_{90}ppm (LCL-UCL)	Slope	r^2
Fr.1	1st	15.45 (13.53–17.64)	63.29 (45.90–105.80)	35	0.892
	2nd	18.36 (17.02–20.90)	79.09 (54.66–145.40)	55	0.789
	3rd	20.40 (17.45–23.86)	76. 63(54.62–132.23)	50	0.888
	4th	–	–	–	–
Fr.2	1st	16.80 (14.31–19.73)	74.70 (51.98–135.71)	35	0.899
	2nd	21.36 (18.28–24.36)	85.73 (58.73–160.27)	45	0.882
	3rd	23.21 (20.79–25.91)	74.81 (54.27–125.35)	30	0.818
	4th	24.50 (22.33–26.87)	96.94 (65.11–192.26)	20	0.898
Fr.3	1st	20.16 (17.36–23.41)	70.65 (51.66–115.99)	15	0.892
	2nd	23.91 (20.79–25.91)	74.81 (54.27–125.35)	30	0.888
	3rd	–	–	–	–
	4th	–	–	–	–
Fr.4	1st	24.50 (19.87–28.61)	61.49 (47.31–93.28)	15	0.872
	2nd	–	–	–	–
	3rd	–	–	–	–
	4th	–	–	–	–
Fr.5	1st	5.74 (4.58–7.20)	12.70 (11.41–14.47)	70	0.999
	2nd	6.14 (6.02–7.44)	15.50 (10.06–37.78)	90	0.982
	3rd	7.07 (5.56–8.99)	21.25 (13.45–227.12)	60	0.981
	4th	11.00 (9.29–13.02)	23.99 (15.13–316.10)	65	0.999
Fr.6	1st	18.51 (16.76–20.47)	56.83 (43.89–84.06)	20	0.828
	2nd	21.36 (18.28–24.96)	64.00 (48.32–98.99)	35	0.888
	3rd	21.49 (18.16–25.42)	61.79 (47.28–93.49)	15	0.878
	4th	24.09 (0.57–28.20)	55.61 (43.95–80.11)	10	0.864
Fr.7	1st	10.88 (9.95–11.89)	27.19 (23.83–32.35)	65	0.899
	2nd	13.83 (12.35–15.48)	42.56 (34.42–57.91)	50	0.880
	3rd	16.37 (14.69–18.24)	47.21 (37.88–65.17)	30	0.875
	4th	17.38 (15.1–19.90)	50.07 (39.99–69.76)	25	0.865
Fr.8	1st	15.49 (13.67–17.56)	57.19 (43.05–88.72)	15	0.875
	2nd	17.78 (15.98–19.78)	54.53 (42.39–79.68)	40	0.870
	3rd	22.36 (19.11–29.16)	69.18 (51.31–111.01)	30	0.855
	4th	24.50 (21.63–28.10)	58.46 (45.59–86.88)	25	0.850
Fr.9	1st	12.01 (10.62–13.59)	39.64 (32.03–54.08)	45	0.885
	2nd	13.69 (11.91–15.73)	58.59 (42.98–95.98)	50	0.875
	3rd	15.84 (14.02–18.01)	62.38 (45.87–101.25)	35	0.875
	4th	18.10 (15.83–20.70)	62.54 (46.82–99.33)	30	0.865
Fr.10	1st	14.54 (12.98–16.29)	47.33 (37.42–67.04)	15	0.880
	2nd	17.48 (15.13–19.68)	65.90 (48.00–108.93)	30	0.870
	3rd	23.36 (20.70–24.16)	46.09 (38.22–61.29)	25	0.864
	4th	–	–	–	–

Control—nil mortality, significant at $p < 0.05$ level, LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, LCL lower confidence limit, UCL upper confidence limit, r^2 regression coefficient, (−) <50%.

and *Hyalomma marginatum isaaci* was LC_{50} = 12.25 and 12.17 mg/L, LC_{90} = 49.17 and 46.52 mg/L.

Synthesized Ag NPs against first to fourth instar larvae of *A. aegypti* and *C. quinquefasciatus* at concentration of 10 μg/mL. The results showed that the optimal hours for measuring the percent mortality were 100, 100, 85, 68 and 42; 100, 92, 80, 60 and 40; 100, 88, 75, 58 and 38; 100, 85, 72, 56 and 35 against 1st, 2nd, 3rd and 4th instar larvae of *A. aegypti* and 100, 100, 80, 63 and 38; 100, 90, 80, 60 and 38; 100, 85, 75, 60 and 35; 100, 82, 70, 53 and 30 against 1st, 2nd, 3rd and 4th instar larvae of *C. quinquefasciatus* at 24, 18, 12, 6 and 1 h. The lethal effect of first to fourth instars larvae *A. aegypti* (LC_{50} = 2.50, 2.78, 3.02, 3.05 μg/mL; LC_{90} = 8.28, 7.47, 8.13, 8.74 μg/mL) and *C. quinquefasciatus* (LC_{50} = 2.75, 3.00, 3.21, 3.48 μg/mL; LC_{90} = 6.45, 7.72, 11.06, 12.83 μg/mL), respectively (Table 3). The control (distilled water) showed nil mortality in the concurrent assay. Larval mosquito control, particularly in sensitive environments, has come to rely heavily on a small number of materials with a high degree of target specificity.

Marimuthu et al. (2011) reported bioactivity of synthesized Ag NPs using *Mimosa pudica* leaf extract tested against the larvae of *C. quinquefasciatus* (LC_{50} = 13.90 ppm). Synthesized Ag NPs using *Tinospora cordifolia* leaf extract tested against the larvae of *C. quinquefasciatus* LC_{50} = 6.96 mg/L (Jayaseelan et al., 2011). Larvicidal activity of synthesized Ag NPs utilizing an aqueous extract from *Eclipta prostrata* was observed in synthesized Ag NPs against *C. quinquefasciatus* (LC_{50} = 4.56 mg/L; LC_{90} = 13.14 mg/L) (Rajakumar and Rahuman, 2011). To the best of our knowledge there is no report in the literature for the control of mosquito population by using synthesized Ag NPs using hydroxyanisole. This is an ideal eco-friendly approach for the control of dengue vector, *A. aegypti* and filariasis vector, *C. quinquefasciatus*.

Mechanisms of toxicity are still poorly understood although it seems clear that in some cases, nanoscale specific properties may cause bio-uptake and toxicity over and above that caused by the dissolved Ag ion (Fabrega et al., 2011). The exact mechanism of the formation of these nanoparticles in these biological media is unknown. Presumably biosynthetic products or reduced cofactors play an important role in the reduction of respective salts to nanoparticles. It seems quite probable that the phenols play an important part in the reduction of ions to Ag NPs as the concept of antioxidant action of phenol compounds is not new.

Chromatographic purification: TLC

The isolated fractions were monitored by TLC until a single spot was obtained. The plates were air-dried and exposed to iodine chamber to locate the spots. Fr.5 showed single band with Rf values of 0.38. The identification and characterization of the purified compounds were elucidated by the analysis of spectroscopic data.

Spectroscopic data of the isolated compound

Column chromatography of the ethyl acetate soluble extract of *C. dactylon* gives hydroxyanisole compound (Fig. 1). ^{1}H NMR

Table 2
Larvicidal activity of *C. dactylon* fractions against *C. quinquefasciatus*.

Extracts	Larvae instars	LC_{50}ppm (LCL-UCL)	LC_{90}ppm (LCL-UCL)	Slope	r^2
Fr.1	1st	18.56 (16.93–20.35)	54.55 (42.62–79.02)	20	0.870
	2nd	22.36 (20.70–24.16)	46.09 (38.22–61.29)	25	0.860
	3rd	23.46 (21.66–25.80)	52.36 (42.04–73.99)	10	0.750
	4th	–	–	–	–
Fr.2	1st	20.16 (17.36–23.41)	84.15 (52.66–202.26)	35	0.860
	2nd	22.36 (19.11–26.16)	88.52 (60.51–166.27)	35	0.855
	3rd	24.75 (21.67–27.37)	88.33 (61.11–164.33)	20	0.850
	4th	25.23 (22.27–26.66)	96.94 (65.11–192.26)	30	0.852
Fr.3	1st	18.89 (16.51–21.61)	83.94 (56.83–161.17)	30	0.865
	2nd	19.68 (17.75–21.81)	94.09 (62.17–189.60)	35	0.872
	3rd	22.36 (19.11–26.16)	129.63 (68.82–490.00)	25	0.842
	4th	–	–	–	–
Fr.4	1st	22.36 (19.11–26.16)	63.58 (48.33–97.53)	35	0.855
	2nd	–	–	–	–
	3rd	–	–	–	–
	4th	–	–	–	–
Fr.5	1st	8.58 (4.99–14.77)	19.93 (11.42–0.00)	50	0.999
	2nd	8.62 (4.95–15.03)	21.01 (8.53–51.73)	70	0.998
	3rd	10.28 (7.46–14.17)	23.77 (9.25–61.07)	65	0.999
	4th	10.20 (8.16–12.74)	24.79 (14.60–0.00)	45	0.987
Fr.6	1st	17.18 (14.88–19.85)	63.61 (43.28–147.07)	30	0.865
	2nd	24.50 (20.36–29.48)	78.70 (52.13–185.67)	15	0.888
	3rd	24.35 (21.67–27.37)	67.88 (48.28–130.48)	10	0.782
	4th	–	–	–	–
Fr.7	1st	11.74 (10.14–13.59)	32.70 (27.19–44.96)	60	0.885
	2nd	14.90 (13.21–16.80)	38.12 (31.32–53.05)	45	0.880
	3rd	17.78 (15.98–19.78)	47.22 (36.70–74.15)	55	0.872
	4th	19.68 (16.84–23.00)	55.62 (36.71–158.11)	40	0.864
Fr.8	1st	16.21 (14.39–18.26)	48.96 (36.90–84.01)	45	0.892
	2nd	21.55 (18.07–25.07)	52.49 (40.70–81.83)	25	0.860
	3rd	22.49 (20.73–24.41)	54.79 (42.08–87.19)	40	0.888
	4th	24.65 (21.63–28.10)	60.84 (45.56–102.74)	35	0.752
Fr.9	1st	14.65 (13.09–16.40)	41.16 (32.68–62.25)	35	0.972
	2nd	18.49 (16.74–20.41)	66.60 (48.70–109.15)	55	0.870
	3rd	19.17 (17.48–21.02)	58.51 (44.92–87.51)	35	0.869
	4th	20.04 (18.16–22.10)	52.51 (41.89–73.66)	50	0.865
Fr.10	1st	17.01 (15.52–18.64)	84.23 (57.42–159.19)	25	0.875
	2nd	21.55 (18.07–25.70)	54.07 (58.30–153.32)	45	0.865
	3rd	23.21 (20.79–25.91)	76.31 (55.10–129.28)	40	0.855
	4th	24.65 (21.63–28.10)	71.36 (52.64–116.56)	20	0.850

Control—nil mortality, significant at $p < 0.05$ level, LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, LCL lower confidence limit, UCL upper confidence limit, r^2 regression coefficient, (−) <50%.

spectrum hydroxyanisole the strong signal observed at $\delta = 3.3–3.8$ ppm is due to CH_2. Signal at $\delta = 5.1–5.3$ ppm could be due to aliphatic OH group, whereas the signals appearing between $\delta = 1.0$ and 1.82 ppm are related to aliphatic C—CH_2C≡groups. It should be noted that no signal appears in the $\delta = 6–8$ ppm and $\delta = 10–12$ ppm ranges in which signals due to aromatic and carboxylic acids are expected. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 184.1 (C-4), 128.3 (C-1) and 54.8 (2 OCH_3).

Table 3
Toxicity of silver nitrate and synthesized Ag NPs against mosquito larvae.

Materials	Species	Larvae instars	LC_{50} μg/mL (LCL-UCL)	LC_{90} μg/mL (LCL-UCL)	Slope	r^2
0.5 Mm ($AgNO_3$) solution	*Aedes aegypti*	1st	60.30 (52.16–69.70)	227.85 (194.52–500.50)	36	0.882
		2nd	69.36 (60.97–78.90)	307.09 (211.79–568.37)	42	0.988
		3rd	76.96 (68.89–85.96)	358.94 (237.76–722.77)	28	0.878
		4th	30.51(24.87–37.43)	338.83 (233.47–625.92)	48	0.888
	Culex quinquefasciatus	1st	68.28 (59.94–77.79)	281.74 (200.12–490.16)	54	0.887
		2nd	77.63 (67.98–88.66)	339.38 (230.11–649.21)	14	0.788
		3rd	84.06 (68.36–103.37)	335.40 (232.31–613.10)	32	0.898
		4th	91.46 (77.92–107.35)	381.46 (255.11–751.23)	44	0.888
Synthesized Ag NPs	*Aedes aegypti*	1st	2.50 (2.09–2.99)	8.28(2.28–30.03)	69	0.989
		2nd	2.78 (2.71–3.50)	7.47 (5.28–18.39)	79	0.999
		3rd	3.02 (2.41–3.78)	8.13 (5.58–25.33)	76	0.988
		4th	3.05 (2.49–3.74)	8.74 (5.84–35.43)	86	0.982
	Culex quinquefasciatus	1st	2.75 (2.25–3.35)	6.45 (4.31–26.85)	63	0.999
		2nd	3.00 (2.45–3.68)	7.72 (5.53–17.59)	79	0.989
		3rd	3.21 (2.66–3.86)	11.06 (8.81–15.84)	75	0.988
		4th	3.48 (2.90–4.18)	12.83 (9.92–19.53)	69	0.999

Control—nil mortality, significant at $p < 0.05$ level, LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, LCL lower confidence limit, UCL upper confidence limit, r^2 regression coefficient.

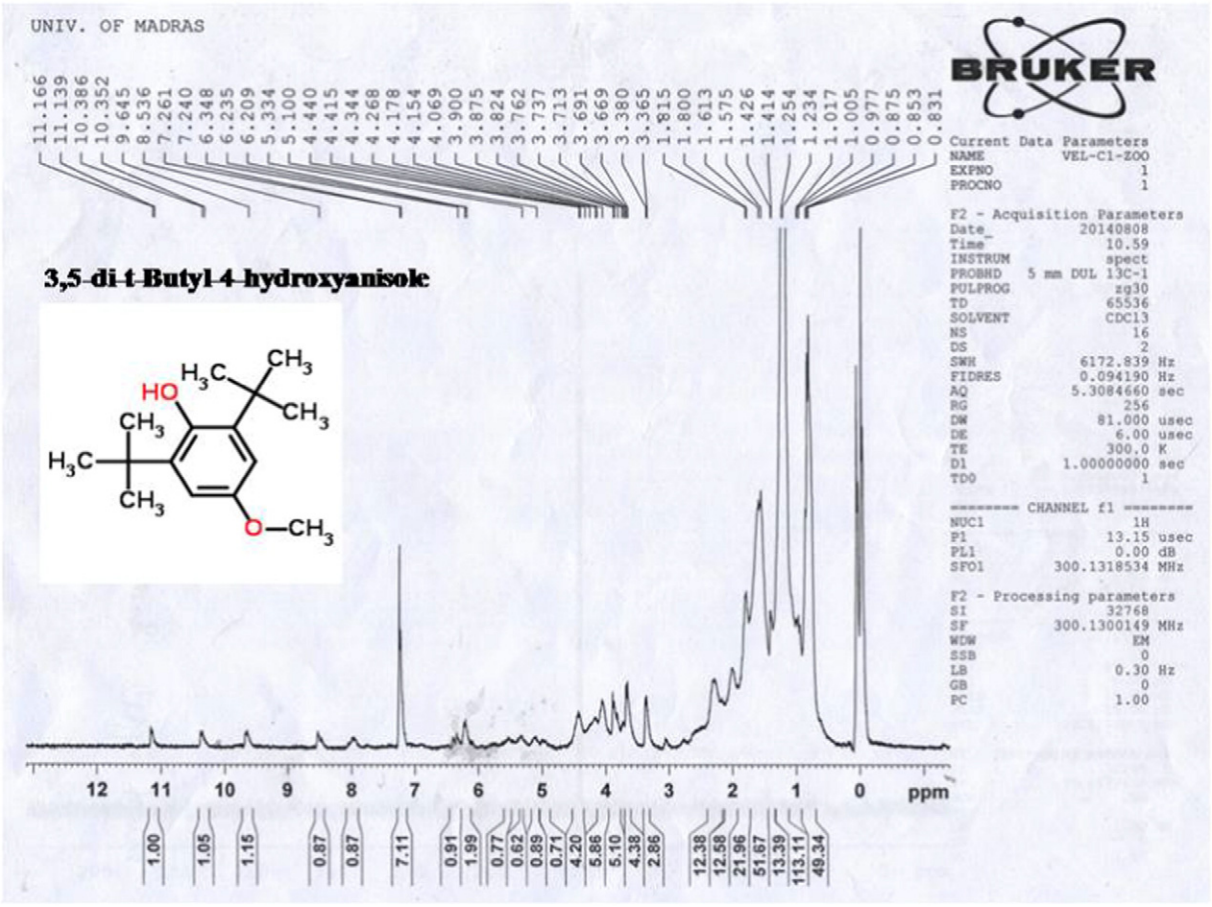

Fig. 1. Chemistry involved in Ag NPs formation ^{1}HNMR.

UV-analysis

The aqueous silver nitrate solution turned to brownish within a few seconds and the intensity increased in direct proportion to incubation period (Fig. 2A). The absorption spectrum of hydroxyanisole surface plasma resonance observed at 418 nm confirms the formation of Ag NPs (Fig. 2B). It may be due to the excitation of Surface Plasmon Resonance (SPR) of the synthesized Ag NPs (Rajakumar and Rahuman, 2011). The SPR band at 410 to 430 nm confirmed the synthesis of Ag NPs at plant products (Sathishkumar et al., 2009). Formation of Ag NPs using 0.5 mM solution of $AgNO_3$ was confirmed using UV– visible spectral analysis.

XRD analysis synthesized Ag NPs

The XRD pattern showed three intense peaks in the whole spectrum of 2θ values ranging from 20 to 80. A number of Bragg reflections with 2 theta values of 39.18°, 43.56°, 53.86° and 70.50° sets of lattice planes were observed (Fig. 3). The sharpening of the peaks clearly indicates that the particles were in the nanoregions. The mean size of Ag NPs was calculated using the Debye–Scherrer's equation by determining the width of the Bragg's reflection. The size of the nanoparticles was thus determined to be about 12 nm for Ag NPs synthesized at room temperature. Kumar et al. (2012) reported the unidentified crystalline peaks (27.89°, 32.30°, 46.26°, 54.79°) are also apparent in many works

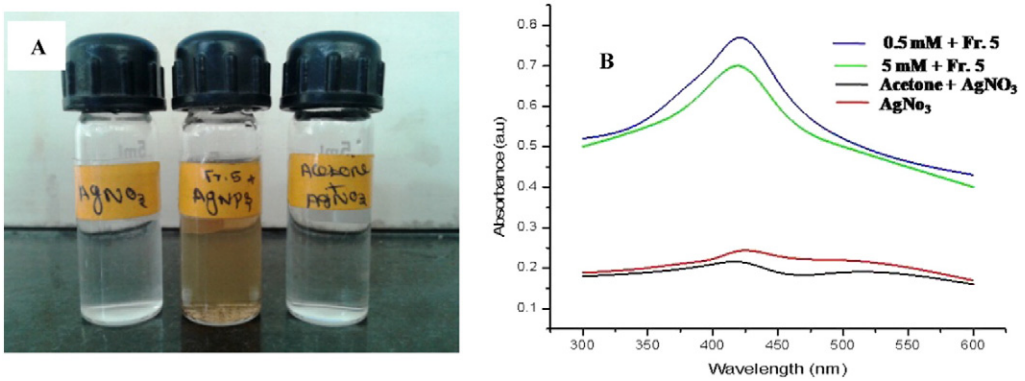

Fig. 2. (A) Color changes during Ag NPs formation (B) UV–visible absorption spectrum of biosynthesized Ag nanoparticles hydroxyanisole.

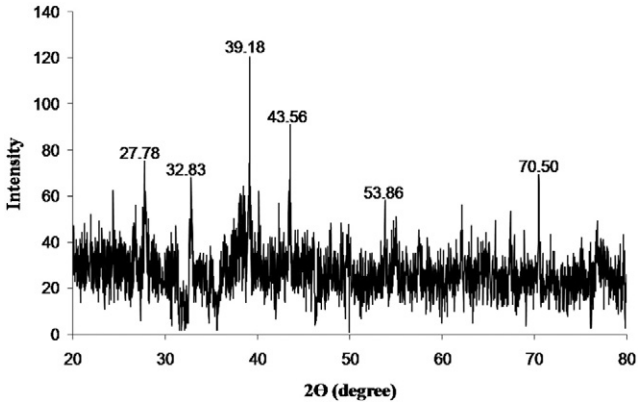

Fig. 3. X-ray diffraction pattern of synthesized silver nanoparticles.

in which the XRD pattern includes the relevant 2θ range. These peaks are due to the organic compounds which are present in the extract.

Nanoparticle composition and size distribution

The size and shape of the synthesized silver nanoparticles were examined using HRTEM analysis. HRTEM images of Ag NPs at different magnifications (Fig. 4). It is clear that the nanoparticles are almost spherical in shape with average size about 14 nm.

Mechanism complicated during Ag NPs formation

Mukhlesur Rahman et al. (2005) investigated that terpenoids are anticipated to play a key role for the reduction of silver ions and the formation of corresponding nanoparticles, while ketones/aldehydes bind to emerging spherical nanoparticles to form large nanotriangles and hexagons. However, biochemical pathways responsible for the production of metal nanoparticles using plants are yet to be elucidated.

The present green synthesis shows that the environmentally benign and renewable source of 3,5-di-*t*-butyl-4-hydroxyanisole used as an effective reducing agent for the synthesis of Ag NPs. This biological reduction of metal would be boon for the development of clean, nontoxic and environmentally acceptable "green approach" to produce metal nanoparticles, involving organisms even ranging higher plants. This is the first report on evaluating larvicidal activity for Ag NPs. Further research on Ag NPs could bring a very promising target drug which can be used for protecting mosquito control.

Conflict of interest statement

We declare that we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2016.06.007.

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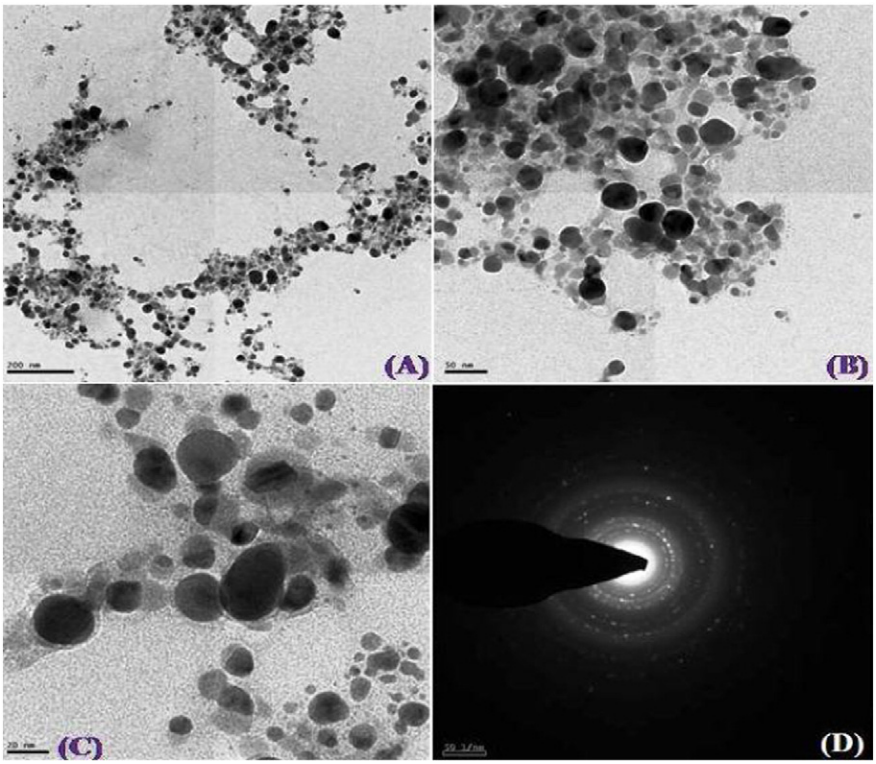

Fig. 4. HRTEM micrograph of synthesized Ag NPs scale bar: (A) 200 nm (B) 50 nm (C) 20 nm (D) selected-area electron diffraction pattern.

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